Protective effects of L-carnitine on the hexachlorophene-induced neurotoxicity and oxidative stress in mice

K. YAPAR1, E. ORUC2, A. KART1* AND M. KARAPEHLIVAN3

1 Department of Pharmacology and Toxicology, College of Veterinary Medicine, University of Kafkas, 36100, Kars, TURKEY.
2 Veterinary Control and Research Institute, 42080, Meram, Konya, TURKEY.
3 Department of Biochemistry, College of Veterinary Medicine, University of Kafkas, 36100, Kars, TURKEY.

* Corresponding author: E-mail: asimkart@yahoo.com - asimkart@hotmail.com

SUMMARY

The aim of this study was to investigate the protective effect of L-carnitine on the hexachlorophene-induced neurotoxicity and lipid peroxidation. Thirty two BALB/c mice were used in this study and were divided into 4 equal groups: animals of the group 1 (control) received daily subcutaneous injections of 0.9 % NaCl for 6 days, animals of the group 2 were treated by L-carnitine (500 mg/kg/day s.c. for 6 days), those of the group 3 received hexachlorophene (126 mg/kg) by gavage and daily injection of 0.9 % NaCl for 6 days and those of the group 4 were treated with L-carnitine plus hexachlorophene. Plasma and brain tissue total sialic acid (TSA), glutathione (GSH), and malondialdehyde (MDA) concentrations were measured 72 hours after the last injection and brain tissue alterations were investigated by conventional histology. Mice treated with hexachlorophene alone (group 3) exhibited higher MDA and TSA concentrations in blood and in brain than those of the 3 other groups whereas plasma and brain GSH concentrations were dramatically depressed. Severe oedema and bleeding were noticed in meninges, cortex, substantia alba and medulla spinalis in this group. By contrast, when animals were exposed to hexachlorophene and L-carnitine, brain injury was markedly reduced. In addition, plasma and brain MDA, TSA and GSH concentrations were significantly modified compared to the group 3. In plasma, the biochemical parameters were similar to values obtained in controls but they remained significantly altered in brain compared to controls. Furthermore, L-carnitine treatment significantly lowered plasma MDA and TSA concentrations and increased GSH concentrations compared to controls. These results evidence the antioxidant properties of L-carnitine and its protective role against the hexachlorophene neurotoxicity and they suggest a beneficial interest of L-carnitine in the treatment of hexachlorophene adverse effects.

Keywords: L-carnitine, hexachlorophene, neurotoxicity, lipid peroxidation, sialic acid, malondialdehyde, glutathione.

Introduction

Hexachlorophene is a phenol compound with a chemical structure as 2, 2 methylenebis (3, 4, 6-trichlorophenol). It is used as a skin antiseptic in newborn humans, and it is also used as an anti-nematode against Fasciola infestations in ruminants and several cestode species in dogs. However, the therapeutic index of this is narrow and over-dosages lead to neurotoxicity [10, 14].

It was reported that hexachlorophene inhibits several enzymes including cytochrome oxidase, lactate dehydrogenase, and succino-oxidase and adenosine 3-phosphatase in the brain, liver, kidney and heart tissue and consequently oxidative phosphorylation [9, 15, 23, 28, 29]. CALDWELL et al. [9] reported that hexachlorophene orally administered to the rats at sub-lethal doses caused the inhibition of oxidative phosphorylation, and that it was the major biochemical toxic effect observed 12-15 hours after treatment. In addition,
hexachlorophene was reported to decrease glutathione concentration and to induce lipid peroxidation in the skin of guinea pigs [27].

Caldwell et al. [9] and Gondolfi et al. [15] reported that death due to chlorophenol intoxication was associated with alterations of oxidative phosphorylation leading to hyperpyrexia which could induce severe damage on several critical biological systems: increases of intracellular Na⁺ and Cl⁻ concentrations were associated with hexachlorophene toxicity in the central nervous system and lead to intramyelinic oedema and spongiosis occur in the cerebrum, cerebellum, liver and kidneys [2, 22]. Intramyelinic oedema and spongiosis occur in the substantia alba of the brain, cerebellum and medulla spinalis [17, 32, 43].

L-carnitine is a vitamin-like cofactor which is structurally similar to the vitamins. Its main function is to serve in the transfer of long chain fatty acids into the mitochondria, in the β-oxidation of fatty acids and in the storage of energy [8, 21]. L-carnitine also serves as a buffer for excess acyl-CoA which could accumulate and lead to cell damage [8]. L-carnitine was reported to be protective for cellular membranes via the detoxification of acetyl groups and free CoA [13] and its antioxidant action on the lipid peroxidation by preventing formation of free radicals [4, 19, 33]. It was also shown that L-carnitine is neuroprotective by improving mitochondrial energy metabolism [7].

Therefore, the glutathione (GSH), malondialdehyde (MDA) and total sialic acid (TSA) concentrations in blood and in brain tissue were measured for evaluating the occurrence of oxidative stress after acute hexachlorophene intoxication while damage of nervous tissue were evaluated by histology.

Material and Methods

1. ANIMALS, SAMPLES AND EXPERIMENTAL PROTOCOL

Thirty two BALB/c mice (12-14 weeks old, approximately 25-30 g) obtained from Konya Veterinary Control and Research Institute were used in this study. The experiment (Protocol #: 3057) was approved by the Institutional Ethics committee of Konya Veterinary Control and Research Institute. All animals were acclimatized for 48 hours and the animals were divided into 4 groups containing 8 mice each. Mice of the group 1 served as controls and received subcutaneous (s.c.) 0.9 % NaCl injection (1ml/day) for 6 days. In the group 2, mice were treated only with L-carnitine (CARNITENE®, Sigma–Tau Industrie Farmaceutiche, Pomezia- Italia) at 500 mg/kg/day s.c. for 6 days. Hexachlorophene (Sigma Chemical Company, St. Louis, MO) was administered via intragastric gavage at the dose of 126 mg/kg along with s.c. 0.9 % NaCl injection (for 6 days) to the animals of the group 3. In the group 4, animals received also daily injections of L-carnitine (500 mg/kg s.c. for 6 days, 3 days before and 3 days after hexachlorophene administration) and single gavage administration of hexachlorophene (126 mg/kg). Under ether anaesthesia, blood samples were collected from the heart via cardiac puncture in EDTA tubes for plasma GSH, MDA and TSA measurements 72 hours after the last drug injection. All tubes were centrifuged (at 1 200 g at 4 °C) for 10 minutes, and plasmas were carefully harvested and stored at -25°C until analysis. In addition, after killing the animals by CO₂ overdose, tissue samples were collected for measurement of GSH, MDA, and TSA concentrations. Brain tissues were immediately removed and washed with 0.15 M KCl (at 4°C), then homogenized in ice-cold 0.15 M KCl by a homogenizer (Ultra Turrax Type T25-B. IKA Labartechne, Germany) at 1 600 rpm for 3 minutes. The homogenates were centrifuged at 5 000 g at 4°C for 1 hour and the supernatants were stored at – 40°C until analysis.

2. BIOCHEMICAL AND HISTOLOGICAL INVESTIGATIONS

Total sialic acid (TSA) was measured colorimetrically using a spectrophotometer (UV-1201, Shimadzu, Japan) by the method of SYDOW [42]. In the analysis of TSA, plasma and brain homogenates were deproteinized by acid per-chloride and then dehydrated by heating with HCl to give rise to furfural derivatives. Furfural derivatives react with p-dimethylbenzaldehyde forming colored product which is read at 525 nm.

GSH concentration was assayed by the method of Beutler et al. [6] based on the spectrophotometry measurement of sulphhydril (-SH) groups forming complexes with 5, 5'- (2-dithiobis nitrobenzoic acid) which give rise to coloured products.

MDA concentrations were carried out by the method of Yoshiko et al. [47] based on reaction between thiobarbituric acid (TBA) and MDA produced as an end product of lipid peroxidation.

For histopathological examinations, brain tissue samples were taken and fixed in 10 % neutral buffered formalin. After the routine histopathology processing, 5-6 μm sections, paraffin sections were prepared and all sections were stained with haematoxylin-eosin (H&E). Histopathological changes were semi-quantitatively assessed under the light microscope with an ocular grid and a 40X objective. A total of 10 high-power fields were randomly chosen. Changes in the experimental histopathological parameters for brain tissue were graded as follows: (-) showing no changes, (+), (++) and (+++) indicating mild, moderate and severe changes, respectively.

3. STATISTICAL ANALYSIS

Differences between the groups were tested by analysis of variance (ANOVA) and Tukey’s test using SPSS for Windows version 10.0. Data were presented as mean ± standard error, and p values less than 0.05 were considered significant.
Results

Plasma and brain MDA, TSA and GSH concentrations obtained in the different groups are presented in Tables I and II, respectively. Plasma and brain TSA and MDA concentrations were significantly increased in mice treated by hexachlorophene alone (group 3) compared to values obtained in the 3 other groups (p < 0.05), whereas plasma and brain GSH concentrations were significantly depressed in this group compared to the others (p < 0.05). Moreover, the L-carnitine treatment (group 2) has induced significant decreases of plasma TSA and MDA concentrations (p < 0.05) and a significant elevation of plasma GSH concentration (p < 0.05) compared to control values. By contrast, no significant variation of brain TSA, MDA and GSH contents compared to controls was noticed in the group 2. When L-carnitine was injected 3 days before and 3 days after the oral hexachlorophene administration (group 4), plasma and brain TSA and MDA concentrations were significantly lower than those obtained in mice treated by hexachlorophene alone (p < 0.05) but remained significantly elevated compared to values observed in mice treated with L-carnitine alone (group 2). Besides, plasma MDA and TSA values were similar to controls. In parallel, mice treated simultaneously by hexachlorophene and L-carnitine presented higher plasma and brain GSH concentrations than mice of the group 3 (p < 0.05) and lower values than mice of the group 2 (p < 0.05).

Histological alterations of brain according to the treatment groups were summarized in Table III. In mice treated by hexachlorophene (alone or in combination with L-carnitine), lesions were observed in meninges (hyperaemia and bleeding), in the substantia alba (bleeding and spongiosis), in brain neurons (chromatolysis and ischemic alterations) and in medulla spinalis. These lesions were dramatically severe in mice of the group 3 (treatment with hexachlorophene alone) (figures 2 and 4), whereas they were notably less intense when mice simultaneously received L-carnitine (group 4) (figure 3). No brain injury was evidenced in animals from the group 1 (control) (figure 1) or from the group 2 (treatment with L-carnitine alone).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>L-Carnitine</th>
<th>Hexachlorophene</th>
<th>Hexachlorophene + L-Carnitine</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA (mg/L)</td>
<td>608.56 ± 15.16b</td>
<td>554.48 ± 14.72c</td>
<td>739.66 ± 14.63a</td>
<td>649.97 ± 14.69b</td>
</tr>
<tr>
<td>MDA (nmol/L)</td>
<td>22.77 ± 1.02b</td>
<td>19.09 ± 0.84c</td>
<td>28.58 ± 1.15a</td>
<td>24.34 ± 1.31b</td>
</tr>
<tr>
<td>GSH (μmol/L)</td>
<td>57.82 ± 1.75b</td>
<td>65.54 ± 1.77a</td>
<td>46.84 ± 1.81c</td>
<td>53.83 ± 1.34b</td>
</tr>
</tbody>
</table>

Different superscripts in the same row indicate significant differences (p<0.05).

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Hexachlorophene</th>
<th>Hexachlorophene + L-Carnitine</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA (mg/L)</td>
<td>4.47 ± 0.20c</td>
<td>4.26 ± 0.15c</td>
<td>5.75 ± 0.18a</td>
<td>5.08 ± 0.22b</td>
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<tr>
<td>MDA (nmol/L)</td>
<td>37.43 ± 2.09c</td>
<td>34.32 ± 1.71c</td>
<td>52.02 ± 1.73a</td>
<td>44.05 ± 1.74b</td>
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<tr>
<td>GSH (μmol/L)</td>
<td>5.70 ± 0.21a</td>
<td>6.21 ± 0.28a</td>
<td>4.26 ± 0.19c</td>
<td>5.02 ± 0.10b</td>
</tr>
</tbody>
</table>

Different superscripts in the same row indicate significant differences (p<0.05).

<table>
<thead>
<tr>
<th>Histological alterations</th>
<th>Control</th>
<th>L-Carnitine</th>
<th>Hexachlorophene</th>
<th>Hexachlorophene + L-Carnitine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meningeal hyperaemia and bleeding</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Haemorrhagiae in substantia alba</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Degeneration (chromatolysis) of neurons</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Spongiosis of the substantia alba in the cerebellum</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Oedema in medulla spinalis</td>
<td>-</td>
<td>-</td>
<td>++</td>
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</table>

Degree of pathological changes: (-) None, (+) Mild, (++) Moderate, (+++) Severe.

Table 1: Plasma malondialdehyde (MDA), total sialic acid (TSA) and glutathione (GSH) concentrations in BALB/c mice 72 hours after treatments: Control, n = 8 (0.9 % NaCl, 1 ml/day s.c. for 6 days), L-carnitine, n = 8 (500 mg/kg, s.c. for 6 days), Hexachlorophene, n = 8 (126 mg/kg via intragastric gavage + 0.9 % NaCl, s.c. for 6 days), Hexachlorophene + L-carnitine, n = 8 (126 mg/kg via intragastric gavage and 500 mg/kg, s.c. for 6 days respectively). Results are expressed as mean ± standard errors. s.c.: subcutaneous injection.

Table 2: Brain malondialdehyde (MDA), total sialic acid (TSA) and glutathione (GSH) concentrations in BALB/c mice according to treatments: Control, n = 8 (0.9 % NaCl 1 ml/day s.c. for 6 days), L-carnitine, n = 8 (500 mg/kg, s.c. for 6 days), Hexachlorophene (126 mg/kg via intragastric gavage + 0.9 % NaCl, s.c. for 6 days), Hexachlorophene + L-carnitine, n = 8 (126 mg/kg via intragastric gavage and 500 mg/kg, s.c. for 6 days respectively). Results are expressed as mean ± standard errors. s.c.: subcutaneous injection.

Table 3: Quantification of histopathological alterations in the treatment groups. Control, n = 8 (0.9 % NaCl 1 ml/day s.c. for 6 days), L-carnitine, n = 8 (500 mg/kg, s.c. for 6 days), Hexachlorophene (126 mg/kg via intragastric gavage + 0.9 % NaCl, s.c. for 6 days), Hexachlorophene + L-carnitine, n = 8 (126 mg/kg via intragastric gavage and 500 mg/kg, s.c. for 6 days respectively).
Previous reports indicate that toxic injury to target cells might be mediated by the disruption of intracellular distribution of Na⁺, K⁺ and Ca²⁺ and water regulation [44]. It has been shown that various types of neuropathies caused by drugs, toxins and diabetes can be mediated via disruption of subcellular element Na⁺, K⁺ and Ca²⁺ composition in the neurons [26]. In the case of hexachlorophene neurotoxicity, intramyelinic oedema and myelin splitting can be related to changes in mitochondrial oxidative phosphorylation. In this study, severe brain and medulla spinalis lesions observed in mice treated with hexachlorophene alone essentially consisted of oedema and bleeding and cell necrosis. Biochemical changes of mitochondria are due to the uncoupling of oxidative phosphorylation [36]. The role of mitochondria in the cell osmoregulation is especially important since mitochondria provide the ATP required driving Na⁺-K⁺-ATPase. Nervous tissue, particularly brain, is greatly vulnerable to the oxidative damage because of its unique properties. Because of the high energy requirement of the nervous system, the functional role of mitochondria is essential and alterations leading to mitochondrial dysfunction could contribute to the generation of superoxide radicals in the cells. In addition, since neuronal membrane lipids contain mainly polyunsaturated fatty acids [11, 18], neurons are especially sensitive to lipid peroxidation. During the normal metabolic events, brain produces hydrogen peroxide, and some excitotoxic amino acids which could be released following lipid peroxidation [18]. Malondialdehyde is an end product produced during lipid peroxidation and is considered as an indicator of lipid peroxidation [11].

Sialic acids are membrane-bound neurominic acids which are found in the oligosaccharide chains of the glycolipids and glycoproteins. In neuronal tissues, sialic acids are located on the glycolipid portion of gangliosides. Serum sialic acid concentrations were found to be increased in cancer patients and several types of inflammatory diseases such as arthritis, Crohn’s disease and psoriasis [38, 41]. Serum sialic acid concentrations are increased in chronic glomerulonephritis, chronic renal failure, chronic liver disease and pneumonia [40]. In addition to MDA, sialic acids could be released from the terminal residues of glycolipids of cell membranes when they were broken during lipid peroxidation [30, 39].

Impairment of oxidant-antioxidant balance in favour of free radicals occurring during some pathological or normal

Discussion

Impairment of oxidant-antioxidant balance in favour of free radicals occurring during some pathological or normal
physiological processes could induce oxidative stress in the cells, although several defence systems including enzymatic and non-enzymatic antioxidant systems could partially protect the cell against oxidative damage. Among these mechanisms, glutathione (GSH) plays an important role in the scavenging of hydroxyl radicals and singlet oxygen [46].

In the present study, mice treated with hexachlorophene alone exhibited the highest MDA and TSA concentrations in brain suggesting the occurrence of oxidative stress and lipid peroxidation in cortex. Plasma and brain GSH concentrations decreased in parallel, showing that this antioxidant was markedly consumed without being regenerated. Besides its lipolytic action, L-carnitine exhibits some antioxidant and scavenging radical properties. The scavenger effect of L-carnitine against reactive oxygen species is due to inhibitory effect on the hydroxyl radical production in the Fenton reaction by chelating iron during the formation of reactive oxygen species [35]. The antioxidant effect of L-carnitine was effectively utilized to prevent toxic effect of several chemicals. For example, cisplatin-induced nephropathy where the oxidative stress and lipid peroxidation are thought to play a major role in the pathophysiology of nephropathy, administration of L-carnitine in Sprague-Dawley rats normalized kidney function by decreasing MDA concentrations and increasing GSH concentrations [37]. In addition, L-carnitine was reported to increase plasma GSH concentrations [12]. Effect of L-carnitine on the GSH concentration is suggested to be associated with number of mechanisms including up-regulation of GSH [1] and also due to increased NADPH generation through increased fatty acid metabolism where NADPH is used by GSH reductase to reduce oxidized GSH (GSSG) to GSH [24].

In this way, plasma MDA and TSA concentrations were significantly decreased whereas plasma GSH concentrations were significantly increased compared to controls in mice treated with L-carnitine alone. Consequently, L-carnitine can attenuate the intensity of an oxidative stress and lipid peroxidation induced by various drugs or aging. ASHRAF et al. [5] have demonstrated a protective effect of L-carnitine on the methamphetamine neurotoxicity, mediated by peroxinitrite radicals. Acetyl-L-carnitine, an esterified form of carnitine, which is involved in the reactions of the oxidative phosphorylation such as p-(trifluoromethoxy)phenylhydrazone [45] and 3-nitropropionic acid (an inhibitor of the succinate dehydrogenase which is involved in the reactions of the oxidative phosphorylation) [7]. In the present study, L-carnitine has obviously protected mice from the hexachlorophene neurotoxicity at least partially, since the L-carnitine treatment before and after hexachlorophene administration has dramatically alleviated brain lesions, has significantly depressed plasma and brain MDA and TSA concentrations and has significantly enhanced plasma and brain GSH concentrations. These results confirm the direct and indirect involvement of L-carnitine in the cellular antioxidant systems and suggest a beneficial effect of L-carnitine in the treatment of neuropathies associated to an oxidative stress, like hexachlorophene neurotoxicity.

References

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